Final synopsis for the proposed Ph. D thesis entitled

Isolation and characterization of microbes of *Stomopneustes variolaris* (Lamarck 1816) from St. Mary's Island, West Coast of India

Submitted by

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Chapter 1: Introduction and review of literature

The marine environment is extremely complex and contains a huge diversity of life forms. The water column of the oceans contains approximately 10^6 bacterial cells per millilitre (Hagstrom *et al.,* 2002). Marine bacteria and fungi are of great interest as novel and rich sources of biologically active products. They live in close association with soft-bodied marine organisms, which lack obvious structural defence mechanisms, and thus rely on chemical defence by production of bioactive secondary metabolites, either by themselves or by associated microflora, to survive in their extreme habitat (Jensen and Fenical, 1994). In the last decades, the number of reported secondary metabolites from marine bacteria and fungi has steadily increased (Blunt, 2009), thus reflecting the growing attention by groups from academia and industry. Fungi, in general, have been liberal wellspring of medications as proved by the seclusion of numerous medications in use such as paclitaxel, camptothecin, vincristine, torreyanic corrosive and cytarabine to give some examples (Sithranga and Kathiresan, 2011). In this light, marine is significant from the point of view of new medications as well as a wellspring of new scaffolds that can be modified further to acquire the ideal activity. Among high-value drug leads highly needed now, new antimicrobials targeting bacteria and fungi that are resistant to current commercially available drugs rank on the top. Natural products are the best source of drug leads for antibiotics: about two-thirds of the FDA-approved antibiotic agents are either natural products or natural product derived (Newman and Cragg, 2007).

Marine bacteria and fungi are of considerable importance as new promising sources of a huge number of biologically active products. Some of these marine species live in a stressful habitat, under cold, lightless and high pressure conditions. Surprisingly, a large number of species with high diversity survive under such conditions and produce fascinating and structurally complex natural products.

Marine natural products from microbial origins were considered as significant sources for the discovery of promising drug leads. Marine fungi were a significant source of microbial origins due to their chemical diversity and rich biological activity. However, only a small fraction of the global fungal population, especially marine fungi, has been sampled. According to the published results for marine microbial natural products from 2010 to 2013, marine derived fungi attributed to 64% marine natural products sourced from microbial origin (Zhao *et al.,* 2013).

Sea urchins

Sea urchins belong to the phylum Echinodermata and class Echinoidea are widely distributed in all the seas from the Arctic to Antarctic regions and uniformly distributed in all tropical and subtropical environments. As abundant herbivores and detritivores in many marine ecosystems they are potential marine shredders (James, 2008). They are prominent part of coral reef and are found in many reef habitats including corals algae and sea grasses (Lessios *et al.,* 2001). These are benthic organism occur at all the ocean depths. These marine animals act as biological indicators to assess the environmental health by their sensitivity to the environmental changes or stresses in their habitat (Parish *et al.,* 2012). Consequently, sea urchin grazing is recognized as an important factor in restoring and maintaining recolonization of corals because it can protect corals from competition with other benthic organisms (Dumas *et al.,* 2007). Coral reef ecosystem is subjected to increasing stresses all over the world. It is predicted that these stresses will become more frequent as a result of ongoing changes, especially climate change and inappropriate land and sea use. Grazing animals, especially sea urchins are known to play a major role in the ecology of coral reef habitats by altering the distribution, relative abundance and species composition of marine algae (Williams and Polunin 2001; Coppard and Campbell 2007). Sea urchins are algal grazers (Bronstein and Loya, 2014) occur in several habitats, such as, rocky intertidal ecosystem, kelp forests as well as coral reef (Davidson and Grupe, 2015). These rock-boring sea urchins are considered to be important ecosystem engineers, as they transform natural rocky substrates into sea urchin pits harbouring assemblages of other benthic organisms, for example, molluscs, crustaceans, echinoderms and sponges (Dumont *et al.,* 2013).

So far, about 1000 species of sea urchins have been identified (Yokota *et al.,* 2002) but only 16 species of sea urchins are harvested for food worldwide. Sea urchins have long been harvested for their gonads (roe). They are popular in Korean and Japanese cuisine and are also a traditional food in Chile. The world annual consumption of sea urchins has been steadily increasing over the last decades and sea urchins are one of the important fisheries in several areas of the world (Pearse, 2006). To date in Sri Lanka, 28 sea urchin species have been recorded (Jayakody, 2012) and no species is used for human consumption, because of unexplored echinoderm resources and lack of market in tropical countries (Salon, 1985; Scheibling and Mladenov, 1987) including Sri Lanka. But *Stomopneustes variolaris* species highly inhabits the southwest region of Sri Lanka, explored the biochemical composition (including the proximate, fatty acids profile, vitamins, and carotenoids), essential trace elements (Fe, Zn, Cu, Ni, and Cr), and biometric parameters and it is the only edible species of sea urchin (Jinadasa *et al.,* 2016). Gonads of

sea urchins also called as roe, are in high demand and economically important food across the world as they have high nutritional value (Matranga and Smolenicka, 2002). *S. variolaris* is one of the edible sea urchin species found in the Indian Ocean. Roe consists of water, protein, lipid, carbohydrate, vitamins, and minerals (Kato and Schroeter, 1985).

Stomopneustes variolaris are dark-coloured, omnivores, warm-water echinoderm species and are widely distributed in the Indo-Pacific tropical and subtropical ocean in shady littoral areas with water depth up to 18 m (Smith and Kroh, 2011; Kroh, 2014). *S. variolaris* are found mostly on the rocks and reefs, they bore into the rocks by their mouth and reside in a cup-like kdepression. They usually found underneath of rocks or crevices that provide protection against predators, and they are more abundant in zones where waves are less strong (Alvarado, 2008). This edible black sea urchin commonly found in Gulf of Mannar which are being consumed as delicious food items from the ancient time by the fishermen communities of Mandapam and Pamban village in Ramnathapuram district of Tamil Nadu (Saravanan *et al.,* 2017).

The present study focused on the edible sea urchin *Stomopneustes variolaris* inhabiting in St. Mary's Island West coast of India. Sea urchins are one of the important fisheries in several areas of the world. Their gonads (Roe) are one of the most delicious food items which have higher nutrient and higher demand in world market. Sea urchins are commonly found in shallow sub tidal rocky bottom, feed on algae and organic matter on sea beds. Marine dwelling microorganisms may enter into the sea urchin body through grazing of algae and other food materials. Now a days, there is a big challenge and scope for the marine drugs isolated from marine bacteria and fungi of marine dwelling animals. Hence, an attempt was made to study the following objectives.

Objectives

1. To analyse the biochemical composition of coelomic fluid, gut, gonad, exoskeleton and spines of sea urchin (*Stomopneustes variolaris)*.
2. To isolate and identify the microbes from coelomic fluid, gut, gonad, exoskeleton and spines.
3. To study the morphological and biochemical characteristics of isolated microbes.
4. Molecular characterization and phylogenetic analysis of isolated microbes.
5. To study the antimicrobial activity of isolated microbes.
6. To isolate the bioactive compounds from isolated microbes.

Chapter 2: Biochemical composition of coelomic fluid, gut, gonad, exoskeleton and spines of sea urchin (*Stomopneustes variolaris)*

Collection of samples

Sea urchin (*Stomopneustes variolaris*) were carefully collected from St. Mary's Island also known as Coconut Island Thonsepar, it is a group of four individual islands namely Coconut Island, North Island, South Island and Daryabahadurgarh Island lying a little to the north of the Malpe port of Udupi District, West coast of India, Karnataka (Latitude 13º 20′ 60″ N; Longitude 74º 40′ 60″ E). The sea urchin samples were collected during the month of mid-January 13th 2016, January 15th 2017 and January 18th 2018 around 2.30 to 3.30 pm at the depth of 15 meter by scuba diving. Sea urchin samples were transferred directly to a sterile plastic bag with seawater and brought to the laboratory and preserved at 4 ºC for the further process.

Sample pre-treatment and processing

Sea urchins were thoroughly washed with running tap water followed by sterile distilled water to remove any loosen associated microbes on the surface of sea urchin. Then they were dissected to separate the coelomic fluid, gut, gonad, exoskeleton and spines.

Gonad (roe) and gut samples were dried in hot air oven at 50-60 ℃ for 24 hours after removing excess of water using blotting paper. Coelomic fluid was collected and preserved in an air tight container. Exoskeleton and spines were shade dried and powdered by using mixer grinder. All the powdered samples were kept in separate air tight containers and stored at 4 ℃ for further study.

Total proteins

Materials and Methods

Dry powdered sample of gut, gonad, exoskeleton and spines and coelomic fluid was used for the estimation of total protein by Lowry *et al.*, (1951). Experiment was conducted in triplicates.

The total protein was calculated by using the following formula:

$$\text{Total protein} = \frac{\text{OD of standard} - \text{OD of sample}}{\text{Weight of the sample}} \times 100$$

Results

Total protein content was high in gonad (18.62±0.025%) followed by coelomic fluid (14.15±0.01%), gut (10.09±0.075%), exoskeleton (3.49±0.318%) and spines samples (2.91±0.037%).

Discussion

Protein is an important biochemical macronutrient and it plays a vital role in determining the texture and quality of animal muscle. High amount of protein in gonads of sea urchin species *S. nudus* and *P. lividus* reported by Hiroshi *et al.,* (1998) and Cruz-Garcia *et al.,* (2000) respectively. Mol *et al.,* (2008) reported that the protein content (12.03 ± 1.26%) in gonad of sea urchin *P. lividus.* Shikov *et al.,* (2012) found that the sea urchin *S. droebachiensis* contain 39.7% protein. Bragadeeswaran *et al.,* (2013) elucidated that the protein content of sea urchin *T. toreumaticus* was found 13.91%. High amount of protein content was recorded in gonads of *S. variolaris* collected from Western and Southern coastal area around the Sri Lanka (Jinadasa *et al.,* 2016). Compared with these studies the protein content in gonad sample of *S. variolaris* was high. Shpigel *et al.,* (2004) stated that the proximate composition of sea urchin has been strongly influenced by food quality, which is present in living environment. Kato and Schroeter, (1985) reported protein and ash content of Red sea urchin (*S. franciscanus*) and purple sea urchin (*Strongylocentrotus purpuratus*), showed variation in values. The proximate composition has been strongly influenced by food quality, which is present in living environment. Protein is the main constituent of the gonads of urchins (except moisture) (Verachia *et al.,* 2012). Male and female gonads are the only organs in sea urchins, which are used to store the nutrients for reproduction. Presence of higher amount of proteins in sea urchin gonads is an advantage as they get high protein diet and gonads can be used for substitutes as an alternative protein sources instead of fish supplements (Fabbrocini and D'Adamo, 2011).

Estimation of Carbohydrates

Materials and Methods

Dry powdered sample of gut, gonad, exoskeleton and spines and coelomic fluid was used to estimate carbohydrates by Anthrone method (Carrol *et al.,* 1956). Experiment was conducted in triplicates.

The carbohydrate content was calculated by using the following formula:

$$\text{Carbohydrate} = \frac{\text{OD of standard - OD of sample}}{\text{Weight of the sample}} \times 100$$

Results

High amount of carbohydrate (18.45±0.05%) was recorded in coelomic fluid and gonad (13.70±0.030 %) and low in gut (9.53±0.112 %) followed by exoskeleton (4.35±0.468%) and spines (3.356±0.049 %) samples.

Discussion

Carbohydrate is an essential component of living cells as it supplies the energy needed for physiological and metabolic processes. Shpigel *et al.,* (2004) reported that the lipid and carbohydrate levels decreased with the maturation of the gonad under natural conditions. Mol *et al.,* (2008) reported a concentration of 2.8% of carbohydrate in the roe of the sea urchin, *P. lividus*. Sea urchins are poor in carbohydrate content, but it has a significant role in body coating and fertilization events (Ghazarian *et al.,* 2010). Bragadeeswaran *et al.,* (2013) recorded 2.15 mg/ml of carbohydrate in the sea urchin *T. toreumaticus* sampled in south east coast of India, which represents a lower carbohydrate content. In the present study the carbohydrate content was less when compared with protein. This may be due to the limitation of carbohydrate and abundance of protein in the natural aquatic food web. Moreover, this low value supports the view that carbohydrate plays an insignificant role in energy reserve in aquatic animals (Kelli *et al.,* 2011).

Fatty acid profiles

Materials and Methods

Methanol extract of gut, gonad, exoskeleton and spines were subjected to GC - MS analysis using Shimadzu scientific GC-MS-TQ8040NX, Japan, equipped with column: SH-Rxi-5Sil MS (Crossbone, similar to 5% diphenyl/95% dimethyl pol) (30 meter length X 0.25 mm ID, 0.25 µm thickness) and Helium was used as carrier gas. EI ionization mode was used to obtain mass spectrum analysis. Ion source temperature was maintained at 320 °C and MS transfer line temperature was 350 °C. Interpretation on mass spectrum of GC-MS was made using the database of National Institute of Standards and Technology (NIST 11 & WILEY 8) having not less than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library (Sathyamurthy *et al.,* 2014).

Results

The fat content of each sample was determined as area percentage value with different peaks and retention time. Among the analysed fatty acids, octadecenoic acid, Hexadeconoic acid, methyl sterate, methyl ester and Undecane was high but cholesterol was low in all the samples. Octadecenoic acid, methyl ester with area % of 19.92 and Hexadecanoic acid and methyl ester with area % of 19.60 were found in gonad. In gut, also, similar compounds were

found (Octadecenoic acid, methyl ester, and Hexadecanoic acid, methyl ester) but with different area % of 25.16 and 18.16 respectively. Fatty acid components such as Hexadecanoic acid (24.18) and Tetradecanoic acid (20.51) were isolated from coelomic fluid, Hexadecanoic acid, methyl ester (19.17) and Octadecenoic acid, methyl ester (13.64) from spines and Hexadecanoic acid, methyl ester (11.96) and Undecane (8.26) from exoskeleton samples.

Discussion

Fatty acids are part of lipid class, widespread in nature, food and organisms, being an important constituent of the membrane cell. They have an important biological function, structural and functional roles, and they represent an important source of energy. Lipids are important energy reserves that can store more energy per unit volume than proteins or carbohydrates (Montero-Torreiro *et al.,* 2003). In addition, lipids such as phospholipids, cholesterol and triglycerides are structural components of cells and subcellular membranes and it is important for the vital somatic growth (Liu *et al.,* 2007). Lipids of marine origin are rich sources of Omega-3- polyunsaturated fatty acids and they have pronounced hypo cholesterolemic effect when supplemented in human diet. Muflihunna *et al.,* (2021) reported 7 active components include 9-Octadecenoic acid (Z) -, methyl ester, Hexadecanoic acid, methyl ester, Octadecanoic acid, methyl ester, 9,12-Octadecadienoic acid (Z, Z) -, methyl ester, n-Hexadecanoic acid, Octadecanoic acid, methyl ester, 9,12-Octadecadienoic acid (Z, Z)-, methyl ester, n-Hexadecanoic acid, Hexadecanoic acid, methyl ester, 9,12-Octadecadienoic acid (Z, Z) Acid, Ethyl Ester, and Ethyl Oleate. The highest percentage of peak area is 9-Octadecenoic acid (Z)-, methyl ester. *Holothuria edulis* contains 3 active components, including [1,1'-Bibicyclo [2.2.2] octane]-4-carboxylic acid, 5- (1-bromo-1-methyl-ethyl) -2-methyl-cyclohexanol, and Hexadecanoic acid, ethyl ester from the crude extracts of sea Cucumber *Holothurian atra* and *Holothurian edulis* by GC-MS analysis.

Detection of Minerals

Qualitative and quantitative analysis of minerals

Materials and Methods

Accumulation of minerals (O, Mg, Al, Si, Cl, K, Ca, Fe, C, S, P, Na, Mn, Co, Cu, Zn) were analysed with dry powdered samples of gonad, gut, exoskeleton, spines and liquid sample of coelomic fluid. Mineral content in sample was detected using EDS system [Carl Zuiess-

Fesem attached with oxford instruments EDAX systems]. The mineral analysis was done in triplicate.

Results

Qualitative analysis showed the both positive and negative results for the tested minerals. Oxygen content was high in all the tested samples (coelomic fluid, exoskeleton, spines, gonad and gut). Sodium content was found to be higher in gonad (5.28%) followed by gut (4.23%), exoskeleton (3.78%), spines (3.64%) and coelomic fluid (1.75%). Magnesium content was high in exoskeleton (12.69%) and coelomic fluid (12.14%) compared to gut, gonad and spines (8.96%, 8.26%, and 6.25% respectively). Sulphur content was high in exoskeleton (3.43%) followed by gut (2.32%), gonad (1.89%), spine (1.38%) and coelomic fluid (1.25%). The amount of chlorine was high in exoskeleton (2.09%) when compared to other samples like gonad (1.54%), gut (1.24%), coelomic fluid (1.25%) and spines (1.12%). Calcium content was found to be higher in exoskeleton (127.38%) and spines (117.44%) followed by gonad (32.63%), gut (19.46%) and coelomic fluid (1.32%). Iron content was high in spines (3.53%) and gut (2.45%) compared to exoskeleton (2.24%) and coelomic fluid (1.56%). Potassium was found only in gut (6.23%) and gonad (4.32%) samples. Manganese content was found only in gut (1.46%) and cobalt in gut (2.08%) and gonad (1.25%) samples. Zinc was observed only in gut (3.47%) and gonad (2.62%) and phosphorus content was very low and recorded only in exoskeleton (1.05%) spines (1.02%) and coelomic fluid (0.12%) samples.

Discussion

Sea foods are excellent sources of Ca, P, I, P, Na, Fe and Zn and importance of Ca, Mg and K in the human nutrition (Gopakumar, 1997; Rajagopal *et al.,* 1998). Normally higher potassium intakes may be beneficial in preventing kidney stone formation. On the other hand, phosphorous helps in kidney function, muscle contraction, normal heart beat and nerve signalling. Pearl shells from South China Sea were rich in Ca, P and Zn contents (Zhenglun 1994). Sodium is an essential nutrient that regulates blood volume, blood pressure, osmotic equilibrium and pH in human body. The overall accumulation of essential trace metals in gonads of *S. variolaris* can be used as a bio-indicator to identify trace metal pollution of that particular area of coastal regions in Sri Lanka (Jinadasa *et al.,* 2016). Many sea urchin species, namely *P. lividus* and other species were used as bio-indicators in the world (Mostafa and Collins, 1995; Soualili *et al.,* 2008). Some gonads are a source of vitamins, minerals and other micronutrients, although the diet of the sea urchin can influence the biochemical composition of the gonads (Chen *et al.,* 2010). Drozdov *et al.,* (2016) reported high amount

of Mg, Fe, Zn, and Rb content in the skeleton of spines and tests of the species of sea urchins *Strongylocentrotus intermedius, Mesocentrotus nudus, Scaphechinus mirabilis,* and *Echinocardium cordatum* from the Sea of Japan. In the four species of sea urchins studied, the strength and other mechanical properties of the tests and spines differ and depend on the chemical composition and structural organization of their components.

Physicochemical parameters

Estimation of moisture and Ash content

Materials and Methods

Pre weighed wet samples such as gut, gonad, exoskeleton and spines were used to determine the moisture content according to the AOAC (1990) method. Experiment was carried out in triplicates.

$$\text{Percentage of moisture} = \frac{\text{Wet weight} - \text{Dry weight X 100}}{\text{Wet weight}}$$

Dry powdered samples of each of gut, gonad, exoskeleton and spines were used to estimate ash content by the following method (AOAC, 1995). Experiment was carried out in triplicates.

$$\text{Percentage of ash} = \frac{\text{Dry weight of the sample X 100}}{\text{Original weight of the sample}}$$

Results

Moisture content was high in gonad (65.06±0.036%) followed by gut (45.43±0.152%), exoskeleton (21.6±0.888%) and spine (19.60±0.28%) samples.

Ash content was high in exoskeleton (2.75±0.404%) followed by spines (1.98±0.01%), gonad (1.84±0.041%) and gut (1.33±0.050%) samples. Experiment was carried out in triplicates.

Discussion

The physicochemical parameters are mainly used in judging the purity and quality of the drug. According to Bassey *et al.,* (2011), knowledge of the moisture content of food stuff serves as a useful index of keeping their qualities. The moisture content of *S. franciscanus* gonads (75.4 % ± 0.51) was observed in sea urchin species (Tolga Dincer and Sukran Cakli, 2007). Ayyagiri and Ramesh Babu (2016) reported moisture (77.53% ± 0.80) and ash content (3.76% ± 0.25) based on dry weight on gonads of sea urchin *S. variolaris.* Ashing is an important tool for detecting the adulteration in crude drugs. Ash is one of the least studied

chemical constituents in marine animals. High level of ash content in exoskeleton may be due to the high level of chitin strengthened by high level of calcium in the sea urchin shell. Ash content (3.73% ± 0.05) of the gonad of *P. lividus* was higher (1.5% ± 0.1) than *S. nudus* (Tolga Dincer and Sukran Caki, 2007). Pais *et al.*, (2011) reported that the ash content in the sea urchin *P. lividus* gonad was 1.88 ± 0.003% to 2.35 ± 0.25%. Study revealed the low ash content in gut compared to other samples analysed. Gokulakrishnan *et al.*, (2015) reported that the protein, carbohydrate, fat, ash, moisture content of three different tissues namely spine, viscera and test region of purple sea urchin *S. virgulata* collected from Mudasalodai, Tamil Nadu, Southeast coast of India.

STATISTICAL ANALYSIS

Triplicate determinations were carried out for each biochemical, mineral and physicochemical analysis. The experimental results were expressed as mean ± Standard Deviation (SD) of triplicates.

Chapter 3: To isolate and identify the microbes from coelomic fluid, gut, gonad, exoskeleton and spines.

Materials and Methods

The gut, gonad, coelomic fluid were then teased and macerated gently with the help of sterile glass rod and forceps in order to release any organisms adhered to the tissue into the surrounding saline and homogenised with sterile saline water. Exoskeleton and spine samples were homogenized in sterile saline water by using mortar and pestle. The individual samples were centrifuged at 8000 rpm for 10 minutes and then the supernatant was collected in separate sterile falcon tubes. 5 mL of each aliquot was diluted to 1:1 with sterile 2.5% saline. This was done in order to enrich the organisms present in the samples since a higher salt concentration would mimic their natural habitat (sea water).

Isolation and identification of bacteria

Spread plate method

To isolate bacteria, 0.1 mL of enriched and unenriched coelomic fluid, gut, gonad, exoskeleton and spines samples were spread on Zobell marine agar (ZMA) plates to obtain marine bacteria and sterile Potato dextrose agar (PDA) plates to obtain marine fungi. 100 µg/mL of Ampicillin was added to the PDA plates in order to avoid bacterial growth. Petri plates (PDA) were incubated for 10-15 days at room temperature until the morphology of the fungi could be distinguished (Diane, 2011).

A lawn of bacterial growth was observed on overnight incubation at room temperature in all the samples (enriched and unenriched) except exoskeleton and spines samples. Therefore, the enriched samples were serially diluted tenfold up to 10^{-6} dilution.

Determination of CFU/mL of the sample

CFU/mL was determined according to the method (Goldman and Green, 2008). The enriched samples of gut, gonads and coelomic fluid were tenfold serially diluted up to 10^{-6}. 0.1 mL of the final three dilutions i.e., 10^{-4}, 10^{-5} and 10^{-6} of each sample was spread on the sterile ZMA plates and incubated at room temperature overnight. Colonies were counted and expressed as colony forming units per mL (cfu mL^{-1}).

Results

Countable colonies were observed on 10^{-4} dilution plates of the gut and coelomic fluid sample but no colonies were observed on gonad sample. Therefore, the lower dilutions of gonad sample were spread. Countable colonies were obtained on the 10^{-2} dilution for the gonad sample. Totally, 3 types of 197 colonies (92 in plate I and 105 in plate II) (1 x 10^7 cfu/mL^{-1}) were observed in coelomic fluid. In gut, 58 colonies with only one type (25 in plate I and 33 in plate II) (3 x 10^7 cfu/mL^{-1}) was observed. 2 types of 98 colonies (32 in plate I and 62 in plate II) were found in gonad samples (5 x 10^4 cfu/$mL^{-1)}$. There were no colonies observed in exoskeleton and spine samples.

Morphologically distinct bacterial colonies were isolated and streaked again on ZMA plates to get clear appearance of bacteria and they were marked as C (Coelomic fluid), G (Gut), Go (Gonad) on Petri plates for easy identification and incubated overnight at room temperature. Finally, pure culture of bacterial colonies was obtained for further study.

Isolation and identification of fungi

0.1 mL of enriched as well as unenriched sample of the coelomic fluid, gut, gonads, exoskeleton and spines were spread on sterile Potato dextrose agar (PDA) plates. 100 µg/mL Ampicillin was added to the PDA plates in order to avoid bacterial growth. The enriched and unenriched samples were spread in triplicates. All the PDA plates were incubated at room temperature for 10-15 days.

Chapter 4: To study the morphological and biochemical characteristics of isolated microbes

Morphological study for identification of bacteria

Colony morphology is an important characteristic of bacterial isolation. Based on the colony morphology and Gram-negative staining, isolated organisms were identified based on the

morphological characteristics such as shape, size, margin, pigmentation, elevation, surface, consistency and opacity according to the method of Bergey's Manual of Determinative Bacteriology (Sutherland *et al.*, 2002).

Gram staining was done according to the procedure (Cappuccino and Sherman, 2002). The isolates were smeared on the slides and heat fixed. The crystal violet dye was added, kept for 1 minute and washed in running water. Gram's iodine was added, kept for 1 minute and washed in running water then the smear was decolorized with 90% ethanol and finally the counter stain safranin was added and after a minute washed in running water. It was observed under the light microscope (Olympus clinical microscope model CH20i, Japan).

Results

Only one type of only one type of colonies was observed in all three samples (B/CGGo1). Colonies were 2-3 mm in diameter, round with an entire margin, slightly convex, opaque and glistening. Colony observed in coelomic fluid sample was marked as B/C2 and were 4-5 mm in diameter, completely transparent just like water droplets, circular with entire margin, butyrous and flat. B/C3 was marked for the colony observed in coelomic fluid sample where colonies were translucent, large with 10-13 mm diameter, irregular shape, flat and butyrous

B/Go4: observed in gonad sample, large with 10-15 mm diameter, entire margin and butyrous consistency.

Microscopic observation of bacterial isolates was as follows:

Gram negative short rods were observed in B/CGGo1and B/Go4 followed by slender gram-negative short rods in B/C2 and gram negative very short rods in B/C3.

Morphological study for identification of fungi

Based on the morphological characteristics, fungi were identified and sub-cultured. The fungal strains were identified on the basis of colour, colony morphology, shape, texture, and microscopic characteristics like the presence of specific reproductive structures, shape of conidia and mycelium. Isolated fungi were subjected to the lacto-phenol cotton blue staining. All the procedures were performed aseptically in order to avoid contamination. Morphological characteristics were determined according to the method described (Bensch *et al.*, 2010).

Results

On incubation of three days, 2 types of small non mycelial colonies were observed. One type was present on all the plates of coelomic fluid, gut and gonad samples (named as F/CGGo1) whereas the other type was observed on coelomic fluid and gonad sample plates of enriched

samples (named as F/CGo2). They were isolated and sub cultured on fresh PDA + Ampicillin plates and incubation was further extended up to 15 days. At 5 days of incubation a greenish brown mycelial colony appeared on gonad sample plate and was named as F/Go3. At 7 days of incubation a dark green coloured mycelial colony appeared on the gut and coelomic fluid sample plates and was named as F/CG4. At 15 days of incubation another dark green coloured morphologically different mycelial colony was observed on gut sample plate and was named as F/G5. No fungal colonies were observed on the exoskeleton and spine samples inoculated in petri plates.

Four morphologically different fungal isolates viz., *Cladosporium oxysporium* (F/Go2) and *Corynespora cassiicola* (F/Go3) from gonad, *Cladosporium cladosporioides* (F/GC4) from gut and coelomic fluid and *Cladosporium colombiae* (F/G5) from gut were observed under the microscope. Pure cultures of identified fungi were maintained aseptically for further study.

Biochemical characteristics of bacterial isolates:

The bacterial isolates were subjected to biochemical analysis according to Bergey's Manual of Determinative Bacteriology (Sutherland *et al.,* 2002). The details of each test are given below.

Indole test:

The isolated organism was inoculated in 1 mL of sterile tryptone water and incubated overnight. After 24 hours, 1 mL of xylene was added to the tube and mixed vigorously and allowed it to stand for 5 minutes. 0.5 mL of Kovac's reagent was added down the side of the tube without shaking. Development of pink colour indicates a positive result.

Methyl Red test

The acidic pH of the medium was detected by adding methyl red indicator to an overnight culture of the test organisms in glucose phosphate broth. Development of pink or red colour indicates a positive test.

Voges - Proskauer test

The test organism was inoculated in sterile glucose phosphate broth and incubated overnight. Added 0.5 mL of α-napthol, mixed well and then 0.5 mL 40% KOH was added. Development of red coloured ring within 5 minutes indicates a positive reaction.

Citrate Utilization

For performing the test, the media are inoculated with the test organisms and incubated overnight. Presence of turbidity in Koser's citrate indicates positive test.

Sugar Utilization/Fermentation test

For this test, organisms were inoculated in a culture media containing a specific carbohydrate at 1% concentration in a peptone water base. The acid production was detected (after overnight incubation) by adding a suitable pH indicator such as Phenol Red and the gas formed was detected using a Durham's fermentation tube.

Catalase test

To determine the catalase production by a bacterium, a few drops of 3% hydrogen peroxide can be added to the culture organisms. If effervescence is observed, the enzyme is catalase positive.

Oxidase test

For this test, a loopful of bacterial growth was placed on a Whatmann filter paper and added oxidase reagent (1% solution of N, N, N', N'- tetra-dimethyl-phenylenediamine dihydrochloride) on it. Species which are positive for oxidase enzyme turn purple whereas negative ones show no colour change. All the tests were conducted in triplicates.

Halophilic nature of bacterial isolates

The bacterial isolates were maintained in a high salt environment (2% - 2.5%) throughout the experiment. Therefore, in order to determine whether these bacteria were halotolerant or true halophiles, they were streaked on regular nutrient agar plate containing 0.5% NaCl.

Limit of halotolerance

In order to determine the limit of halotolerance, the four isolates were inoculated in a series of tubes containing nutrient broth with higher NaCl concentrations viz. 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5% and 6%. These tubes were incubated overnight at room temperature.

Results

All the bacteria isolated from coelomic fluid, gut and gonad samples have shown positive result for all the biochemical tests except Voges - Proskauer test. All the bacterial isolates were found oxidase positive but (B/Go4) isolated from gonad showed oxidase negative. All the isolates produced acid only from glucose. Hence, only 95% of the bacterial isolates are oxidase positive. From the results of these tests, the bacterial isolates were confirmed to belong to the genus *Vibrio*.

Except (unidentified) (B/C2), all other isolates could grow at lower salt concentration. Thus, this was the only true halophile. A dense growth was observed in all the cultures tubes. Thus, these bacteria were concluded to be highly salt tolerant.

Discussion

Bacteria belonging to the genus *Vibrio* consist of facultative aerobic and anaerobic gram-negative species, which have a curved morphology, and are generally halo dependent. *V. neocaledonicus*, has been reported on for its corrosion inhibiting effect and this result is comparable with some industrial coatings, since this bacterium affects corrosion by the formation of an inhibitory layer on the metal surface (Gunde-Cimerman *et al.*, 2004; Gunde-Cimerman *et al.*, 2000).

Halophilic bacteria rely on the cellular accumulation of organic osmolytes to prevent dehydration in osmotic environments, such as seawater (Da Costa *et al.*, 1998; Burg and Ferraris 2008). Strategies for osmolyte accumulation were reported for *Enhygromyxa salina* SWB007 and *Pleciocystis pacifica* SIR-1T. While *P. pacifica* accumulates amino acids to offset osmotic stress, *E. salina* produces the ubiquitous osmolytes betaine and hydroxyectoine. Interestingly, all of the sequenced myxobacterial halophiles possess a betaine/carnitine/choline transporter (BCCT) or Bet T homolog, while the sequenced terrestrial myxobacteria do not, regardless of the reported halotolerances (Moghaddam *et al.*, 2016).

Chapter 4: Molecular characterization and phylogenetic analysis of isolated microbes

Molecular characterization and identification of bacterial isolates

Materials and Methods

DNA was extracted from the bacterial isolates by described method (Cheng and Jiang, 2006)

The bacterial isolates were identified using 16S rDNA. For bacterial isolates, a large-scale colony PCR reaction of 100 µl was set up using specific primers to obtain a product of 709 bp. 16S forward primer sequence- 5'GTG TAG CGG TGA AAT GCG 3' and 16S reverse primer sequence- 5' ACG GGC GGT GTG TAC AA 3’ were incorporated in the reaction mixture. PCR products were purified and sequenced using a PCR purification kit (Cosmo Genetech, Republic of Korea), the purified PCR products were reconfirmed (using size marker) by electrophoreses on 1% Agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleosides (dd NTPs) in the reaction mixture (Azwai *et al.*, 2016).

Molecular characterization and identification of fungal isolates

Fungal DNA extraction

Fungal genomic DNA extraction was done according to the method described (Lai *et al.*, 2007). Fungal isolates were transferred onto a new corresponding agar plate based on their morphological differences, growth characteristics, mycelia, and diffusible pigments. Then the plates were incubated at 10 ºC to obtain the pure cultures. Fungal isolates were identified by comparing the morphological observations with internal transcribed spacer sequences (ITS). From the genomic DNA, nearly full-length ITS sequences were amplified by polymerase chain reaction (PCR) using the following primers: ITS1 (50 TCC GTA GGT GAA CCT GCG G -30) and ITS4 (50 -TCC GCT TAT TGA TAT GC -30) (Li, 2009). PCR amplification conditions using ITS1 and ITS4 primers were as follows: the initial denaturation was 7 min at 94 ºC followed by 30 cycles of denaturation for 30s at 94 ºC, annealing for 30s at 55 ºC, extension for 30s at 72 ºC and the final 7 min extension at 72 ºC (Chuanping *et al.,* 2005). 1 % agarose gel was used for the diffusion of PCR products and visualized by ethidium bromide. Each band on a gel electrophoresis represented the single species of fungi. The results obtained on sequencing were uploaded on the NCBI website and BLAST program was run to identify the fungi. Fungal ITS gene sequencing and identification was done according to the following method (Zhang *et al.,* 2012). By sequenced data a phylogenetic tree was constructed using the Clustal Omega tool software, in Molecular Evolutionary Genetics Analysis Tool (MEGA X) (Kumar *et al.,* 2018).

Results

Bacterial isolates were identified as *Vibrio alginolyticus* strain G16 (B/CGGo1) *Vibrio* sp. SOVt012 (B/C2) *Vibrio parahaemolyticus* 3KC1 (B/C3) *Vibrio neocaledonicus* LZ4 (B/Go4).

The isolated fungal species were confirmed by ITS sequencing method. Fungal strains such as *Cladosporium cladosporioides* SCS10 isolated from gut and coelomic fluid (F/GC4), *Cladosporium oxysporium* CBR23 (F/Go2) and *Corynespora cassiicola* ABS48 (F/Go3), *Cladosporium colombiae* DFFSCS017 (F/G5) from gut.

Discussion

Vibrio species are commonly associated with polluted water, seafood, and other aquatic animals as the main source of contamination (Sutherland and Varnam, 2002). *Vibrio parahaemolyticus* inhabits marine and estuarine environments (Rebaudet *et al.,* 2013). Food

borne infections with *Vibrio* spp. are common in coastal cities where retail markets are close to the sea basin (Tena *et al.*, 2010). *Vibrio* species are ubiquitous and abundant in aquatic environments. They exist suspended in the water column, attached to plankton, and in the tissues or organs of various marine animals (Belkin and Colwell 2005). The genus *Vibrio* includes more than 70 species, which are characterized as halophilic or non-halophilic according to their need for sodium chloride for growth (Barbieri *et al.*, 1999; Thompson *et al.*, 2004).

(Tamar Kokashvili *et al.*, 2015) isolated 10 clinically important *Vibrio* species in the aquatic environment of Georgia. *V. parahaemolyticus*, a halophilic *Vibrio* species, mainly causes gastroenteritis associated with consumption of contaminated seafood. In the last decade, epidemics of gastroenteritis in Southeast Asia, Japan, and North America have been linked to *V. parahaemolyticus* pandemic serotype O3:K6 (DePaola *et al.*, 2003). *V. alginolyticus* is commonly associated with ear infections, but can also cause respiratory infections and bacteremia (Uh *et al.*, 2001).

Cladosporium is a member of the form class Deuteromycetes, family Cladosporiaceae under the order Hyphomycetes. It is one of the largest and most heterogeneous genera of hyphomycetes. *Cladosporioids* are common and distributed worldwide (Dinesh, 2019). Several endolithic *Aspergillu*s sp. *Penicillium* sp. and *Cladosporum* sp. (among others) were isolated in 15 reef building corals and hydrozoans from the Caribbean and Australia (Kendricks, 1982). Endophytic fungal strain such as *Cladosporium tenuissimum*, *Cladosporium cladosporioides* and *Cladosporium* sp. were isolated from the Red Sea sponge *Niphates rowi* (Dai, 2006). *Cladosporium* sp. has been recorded from the South China Sea and from marine sponges of Antarctic (Xiao-Yong *et al.*, 2009). Marine fungi have been isolated from macroalgae from maritime, Antarctica (Laura *et al.*, 2018). Fungal species including *Cladosporium cladosporioides* derived from marine animals of Antarctica and sand and seawater of coastal regions of Red Sea and respectively (Valeria *et al.*, 2012; Suaad, 2017). *Corynespora cassiicola* is an Ascomycetus fungi found from a piece of fresh tissue from the inner part of an unidentified sponge collected from the Xisha Islands coral reef in the South China Sea (Dong, 2016). *Cladosporium* species including *Cladosporium oxysporum* was isolated from a natural mangrove environment in Zhanjiang Bay, Guangdong Province, China and they showed pathogenic effects on the mangrove plants (Liu *et al.*, 2017).

Qualitative analysis of Amylase activity of bacterial and fungal isolates

Materials and Methods

Bacterial and fungal isolates were subjected for amylase activity according to DNS method (Miller, 1959).

To perform this assay, bacterial and fungal isolates were streaked on the sterile starch agar plates and incubated overnight at room temperature. Iodine solution was added on to 24 hr bacterial culture and to 5 days fungal culture plates to check for if the bacterial and fungal species produced amylase enzyme.

Determination of Optimum parameters for enzyme activity

The amylase enzyme assay was carried out at three incubation temperatures 10 °C, 30 °C and 50 °C, and also at three pH 6.0, 7.0 and 8.0 in order to determine the optimum temperature and p^H for enzyme activity for both bacterial and fungal isolates of sea urchin.

Results

Colourless zones around the colonies (as compared to blue colour in the surrounding agar) indicated the absence of starch, thus implying that the organisms produced amylase. only *Vibrio neocaledonicus* showed the presence of amylase and other bacterial isolates showed negative result. But all the fungal isolates **(***Cladosporium cladosporioides, Cladosporium oxysporum* and *Cladosporium colombiae* and *Corynespora cassiicola*) showed the presence of amylase production.

Amylase activity of *Vibrio neocaledonicus* was 56±1µg/mL/min. at p^H 7. Fungal species such as *Cladosporium cladosporioides* and *Corynespora cassiicola* were able to produce the amylase enzyme of 54±2.645 µg/mL/min. and 43.66±1.52 µg/mL/min. respectively at p^H 7. *C. Cladosporium colombiae* (78±1µg/mL/min.) and *Cladosporium oxysporum* (82.33±2.51 µg/mL/min.) produced amylase at 8 °C. From these results it was concluded that the optimum p^H for the enzyme was 7.0 whereas optimum temperature was 50 °C. This point should be noted as a thermostable enzyme was obtained from a bacterium whose optimum temperature for growth was 20-25 °C.

Discussion

Amylase activity of *Vibrio alginolyticus* isolated from environment and diseased aquatic animals reported by (Supansa Bupna *et al.*, 2016). Thus, based on the literature as well as the

fact that the high enzyme activity was seen in the cell free broth, we can say that the darker band might belong to amylase. Amylase production in *Cladosporium cladosporioides* isolated from legume seeds collected in Almadinah Almunawwarah, Saudi Arabia by Saleem and Mohsen (2014) and from maize grains (Camila Agnes *et al.*, 2015). Amylase is one of the most important enzymes and of vast significance in present day biotechnology and having approximately 25% of the enzyme market. Amylases are employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents. To convene the demand of these enzymes in industries, a low cost medium is vital for the fermentation of amylase (Sathya and Ushadevi, 2014). Marine microbes as a valuable resource for brand new industrial biocatalysts (Homaei *et al.*, 2016; Beygmoradi and Homaei, 2017).

Chapter 4: To study the antimicrobial activity of isolated microbes

Materials and Methods

Identified bacterial and fungal species and fresh samples exoskeleton, spines and coelomic fluid were subjected for antimicrobial activity against human pathogens.

Test organisms

Human bacterial pathogens such as *Salmonella typhi* (ATCC733), *Streptococcus pyogenes* (ATCC19615), *Escherichia coli* (ATCC7410), *Enterococcus faecalis* (ATCC29212) and fungal pathogens such as *Malassezia globosa* (ATCC14521) and *Candida albicans* (ATCC60193*)* were used as test organisms. The test organisms were obtained from the Institute of Microbial Technology, Chandigarh, India (IMTECH).

Minimum Inhibitory Concentration (MIC) of bacterial and fungal isolates

Microdilution method

MIC is usually considered as the most basic laboratory measurement of the activity of antimicrobial agent against microorganisms. The MIC was considered as the lowest concentration able to inhibit any visible bacterial/fungal growth (Shahidi, 2004). Minimum concentration of drug giving 50% inhibition of OD as compared with control (Clinical and Laboratory Standards Institute (CLSI 2000).

Results

MIC was calculated based on the minimum percentage inhibitory activity (50%) of bacterial and fungal isolates against test organisms at lower concentrations viz., 6.25%, 12.5% and 25%.

MIC of *C. oxysporum* CBR23 against *S. typhi* ATCC733, *E. coli* ATCC7410, *E. faecalis* ATCC29212, *S. pyogenes* ATCC19615, *C. albicans* ATCC60193 and *M. globosa* (ATCC14521) was 12.5%, 25%, 6.25%, 25%, 6.25% and 25% respectively. *V. alginolyticus* G16 showed MIC against *E. coli* ATCC7410 (6.25%,), *C. albicans* (25%,), *E, faecalis* ATCC29212 (12.5%), *S. pkimyogenes* ATCC19615 (25%,), *S. typhi* ATCC733 (6.25%) and *M. globosa* (ATCC14521) (25%). MIC of *C. colombiae* DFFSCS017 was 6.25% (*E. coli* ATCC7410), 25% (*C. albicans*), 6.25% (*E, faecalis* ATCC29212), 25% (*S. pyogenes* ATCC19615), 12.5% (*S. typhi* ATCC733) and 25% (*M. globosa* (ATCC14521)). *V. neocaledonicus* LZ4 showed MIC against *E. coli* ATCC7410, *C. albicans, E, faecalis* ATCC29212, *S. pyogenes* ATCC19615, *S. typhi* ATCC733 and *M. globosa* (ATCC14521) at the concentration of 25%, 12,5%, 6.25%, 25%, 6.25% and 12.5% respectively. *C. cladosporioides* SCS10 showed MIC at 25% against all the test organisms. *V. parahaemolyticus* 3KC1 and *C. cassiicola* showed 50% inhibition at the concentration of 25% and 50% against all the test organisms. Exoskeleton and spines showed 50% inhibition at the concentration of 25%, and 50% against the test organisms. But coelomic fluid showed less than 50% of inhibition at all the concentrations against the test organisms. Ciprofloxacin and Itraconazole were used as standard drug for bacteria and fungi respectively. From the above data, it was concluded that only *V. neocaledonicus* LZ4, *V. alginolyticus* G16, *C. oxysporum* CBR23 and *C. colombiae* DFFSCS017were able to show 50% inhibition against the test organisms at the lower concentrations.

Discussion

Microbial natural products are not only the most reported for their structural novelty, but they also have a wide array of biological properties with targets that are involved in numerous diseases, such as antibiotics, immunosuppressants, anti-diabetics, hormone (ion-channel or receptor) antagonists, anti-cancer drugs, and even as agricultural agents. There was also a strong focus on fungi as a source of active compounds, with 80% of publications focusing on this area (Lalueza *et al.,* 2011). Weiwei Sun *et al.,* (2019) reported that endophytic organisms featured heavily, with endophytes from mangroves, marine invertebrates, and marine algae making up more than 60% of the microbial strains. Some studies have reported that *Vibrio* bacteria could be the producers of biologically active compounds with interesting antibacterial, anticancer and antiviral properties (Maria *et al.,* 2011). Ethyl acetate extract of endophytic fungus *Cladosporium oxysporum* isolated from *Aglaia odorata* Lour. showed antimicrobial activity against *E. coli, S. aureus,* and *C. albicans. Cladosporium oxysporum*

from *Aglaia odorata* could be a good source of antimicrobial substance. It produces bioactive agent that can be developed into a new drug at a larger commercial scale (Noor *et al.*, 2016). Methanolic extracts of *Cladosporium cladosporioides* isolated from the mangrove plant *Sonneratia apetala* (Buch-Ham) from the Sundarbans mangrove forest showed antimicrobial activity against *E. coli* NCTC 12241 and *C. albicans* ATCC 90028 (Tauhidur *et al.*, 2020).

Chapter 5: To isolate bioactive compounds from isolated microbes

Isolation of bioactive compounds by GC-MS method

Materials and Methods

Only *Vibrio alginolyticus* G16 and *Vibrio neocaledonicus* LZ4 (bacteria) and such as *Cladosporium oxysporum* CBR23 and *Cladosporium columbiae* DFFSCS017 (fungi) showed 50% inhibition at lower concentrations against test organisms. Hence, methanol extracts of these species were subjected to isolate the possible secondary metabolites by GC-MS method.

Results

GC-MS showed that the presence of different useful bioactive compounds in these marine bacteria and fungi with different peaks and area %. *Cladosporium oxysporum* CBR23 showed high amount of Methylpalmitate with area % (20.41) and 1- Tridecene (17.35). Tetradecanoic Acid, 12-Methyl-, Methyl Ester compounds 2-propyldecan-1-OL (23.939) were high in *Cladosporium colombiae* DFFSCS017 (28.482). In *Vibrio alginolyticus* G16 Octadecane (28.388) and Methyl Lignocerate (28.493) were found high. *Vibrio neocaledonicus* LZ4 showed high amount of Tetradecanoic Acid, 12-Methyl-, Methyl Ester (32. 274) and 9-Dodecenoic acid, methyl ester (31.922).

Discussion

Marine natural products play an increasingly important role in biomedical research and drug development, either directly as drugs or as lead structures for bioinspired chemical drug synthesis (Molinski *et al.*, 2009). Many marine natural products, especially those isolated from macroorganisms, have already undergone clinical trials (Newman and Cragg, 2007). During the last decades, however, repeated isolation of known metabolites and a reduced hit-rate of novel compounds from marine macroorganisms were observed. Hence, natural product chemists are turning their interest to so far less investigated drug sources, such as marine fungi and bacteria, which turned out to be a vast untapped reservoir of metabolic diversity. Thus, research on chemistry of natural products derived from marine

microorganisms has increased tremendously in recent years due to the demand for compounds having potential pharmaceutical applications or economical value as cosmetics, drugs, fine chemicals and functional personal-care products (Andersen and Williams, 2000).

Even *Vibrio* bacteria are dominant in the marine environment with genomic flexibility, and they have not been explored extensively. Some studies have reported that *Vibrio* bacteria could be the producers of biologically active compounds with interesting antibacterial, anticancer and antiviral properties (Maria *et al.,* 2011). Marine bacteria are prolific producers of such secondary metabolites as they thrive in harsh oceanic climates. Bacteria not only produce secondary metabolites against other organisms, but they also produce certain compounds which help in cleaning their environment. Certain marine bacterial species are known as prolific producers of biosurfactants, bioemulsifiers and exopolysaccharides (Das *et al.*, 2009). Bioactive compounds and their enzyme inhibitory activity have been reported from marine bacterium *Vibrio neocaledonicus* (Isabel *et al.,* 2019).

Marine fungi have been extensively studied and represent a huge portion of antibacterial compound producers among the members of marine microflora. Number of biologically active compounds have been reported from marine fungus *Cladosporium* sp. F14 (Vivek and Bajpai, 2016). Marine-derived fungi have been recognized as one of the tapped sources for new biologically active secondary metabolites including antitumor, antibacterial, antiviral, antifungal, anti- inflammatory and anticancer activities and enzyme inhibitor compounds. Clodepsipeptide isolated from the marine fungus, *Clonostachys* sp. is having anticancer activity (Samuel *et al.,* 2011). Marine fungi have been explored to a much lesser ex-tent than their terrestrial counterparts, such as those for use in treatment of human diseases as well as several others in biotechnological applications. Until 1991, only 321 species of obligate marine fungi had been described, of which 11 belong to the class Ascomycete, which are found in shallow waters. Facultative marine fungi have been explored to a lesser extent, and only 56 species have been described until 1999 (Aline *et al.,* 2008). Between 2000 and 2005, approximately 100 marine fungal metabolites were described and between 2006 and 2010, a total of 690 natural products were reported as being isolated from fungi in marine habitats (Katia *et al.,* 2012). Marine fungi have attracted great attention as considerable resources from only few decades. Recent investigations on marine filamentous fungi looking for biologically active secondary metabolites indicate their tremendous potential as a source of new medicines even at low concentrations of their secondary metabolites (Swathi *et al.,* 2013). A number of biologically active compounds with varying degrees of action, such as

antitumor, anti-cancer, anti-microtubule, anti-proliferative, cytotoxic, photo protective, as well as antibiotic and antifouling properties, have been isolated to date from marine sources (Kalyani and Hemalatha, 2016).

Human impact of isolated microbes

Vibrio spp. are responsible for the majority of human diseases attributed to the natural microbiota of aquatic environments and seafood the most common pathogenic species are *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio alginolyticus* (Shah Faruque, 2006). Cases of *Vibrio* spp. infections have a marked seasonal distribution, with most cases occurring during warmer months. *Vibrio* spp. infections are usually initiated from exposure to contaminated water or consumption of raw or undercooked contaminated seafood and cause a variety of symptoms in humans (Altekruse, *et al.,* 2000). *Cladosporium* species are among the most common fungal inhabitants worldwide, being isolated from almost any environmental source and geographic location (Bensch *et al.,* 2012). The most common *Cladosporium* species are primarily isolated from soil and plant material, where they are frequently encountered as saprobes or secondary invaders on follicular lesions, concomitant with other plant-pathogenic fungi. However, several species are important pathogens of plants and some are also able to affect animals, including humans (De Hoog *et al.,* 2011; Mercier *et al.,* 2013). *Cladosporium* is usually associated with allergic rhinitis (Sellart *et al.,* 2007) or localized superficial or deep lesions (Sang *et al.,* 2012) but, rarely, can cause disseminated infections (Marcelo *et al.,* 2015). *Corynespora cassiicola* is a fungal phytopathogen found in many plant species (Deon *et al.,* 2012). It has been found to infect diverse parts of plants such as leaves, stems, and roots (Shrestha *et al.,* 2017). Although it has been defined as plant pathogen, it opportunistically infects humans and rarely causes phaeohyphomycosis (Zhaolu *et al.,* 2018).

Chapter 6: Summary and Conclusion

Physicochemical and biochemical composition of gut, gonad, coelomic fluid, exoskeleton and spines showed promising results for nutritional quality of edible sea urchin *Stomopneustes variolaris.* The microflora associated with *Stomopneustes variolaris,* especially the fungi and bacteria associated with the gut, gonad and coelomic fluid showed significant results for antibacterial and antifungal activities. Particularly bacterial species such as *Vibrio alginolyticus* G16 and *Vibrio neocaledonicus* LZ4 and fungal species such as *Cladosporium oxysporum* CBR23 and *Cladosporium columbiae* DFFSCS017 showed 50%

inhibition at lower concentrations (MIC) against test organisms. GC-MS analysis showed that the presence of different useful bioactive compounds in these marine bacteria and fungi with different peaks and area %. *Cladosporium oxysporum* CBR23 showed the presence of high amount of Methyl palmitate with area % (20.41) and 1- Tridecene (17.35). Tetradecanoic Acid, 12-Methyl-, Methyl Ester compounds 2-propyldecan-1-OL (23.939) were high in *Cladosporium colombiae* DFFSCS017 (28.482). In *Vibrio alginolyticus* G16 Octadecane (28.388) and Methyl Lignocerate (28.493) were found high. High amount of Tetradecanoic Acid, 12-Methyl-, Methyl Ester (32. 274) and 9-Dodecenoic acid, methyl ester (31.922) was found in *Vibrio neocaledonicus* LZ4.

Based on the present study, we can conclude that isolated microflora from *Stomopnuestes variolaris* indicates the zoonotic significance which can pose substantial concern in aquatic animals and humans since it is consumed as delicacy in Japanese, France, Korean and Mediterranean cuisines. The isolated fungal flora are well known plant pathogens can cause serious impact on aquatic animals including sea urchin as they are potential marine shredders and kelp feeders. But isolated microbes were active against tested human pathogens that may be due to the presence of active compounds. Since the marine bioactive compounds have great value in pharmaceutical industries and cosmetology, they can be potential sources for further biotechnological application.

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PUBLICATIONS

1. Ravitheja S and Chandra M (2019). Isolation and characterization of *Vibrio* Spp. by sequencing of 16s rDNA from coelomic fluid of sea urchin (*Stomopneustes variolaris*). *International Journal of Pharmacy and Biological Sciences,* 9 (1), 330-336.
2. Ravitheja S and Chandra M (2020). Molecular characterization of fungi isolated from sea urchin *Stomopneustes variolaris* (Lamarck, 1816) - St. Mary's Island, West Coast of India. *Biomedicine,* 40(3), 313-318.

PRESENTATIONS

1. Ravitheja S and Chandra M. (2019). Enzyme Analysis of *Vibrio* sp. isolated from gonad sample of sea urchin *Stomopneustes variolaris*. Multidisciplinary International conference on **"Trends in Global Research in Information Technology and their Impacts on Environment"** Organized by Sri Jagadguru Renukacharya College of Science, Arts and Commerce (Bengaluru Central University) Bengaluru, Karnataka, 3rd August, 2019- In Association with TOUCAN Research and Development, Dhanbad (Jharkhand). (ORAL).
2. Ravitheja S and Chandra M. (2017). Bacterial flora of the sea urchin *Stomopneustes variolaris* (Lamarck 1816) from St. Mary's Island, West Coast of India. Two day National conference on **"Reaching the unreached through science and technology: Recent advances in physical, chemical, mathematical and biological sciences for energy, health and environment"** organized by The Indian Science Congress association Bangalore chapter, held on September 8-9, 2017 at Mangala auditorium Mangalore University, Mangalagangothri. (POSTER).